[Plant Signaling & Behavior 4:2, 94-99; February 2009]; ©2009 Landes Bioscience

Review

Very long chain fatty acid and lipid signaling in the response of plants to pathogens

Sylvain Raffaele,† Amandine Leger and Dominique Roby*

Laboratoire des Interactions Plantes-Microorganismes (LIPM); UMR CNRS-INRA 2594/441; Castanet-Tolosan, France

†Present address: The Sainsbury Laboratory; John Innes Center; Norwich, UK

Abbreviations: VLCFAs, very long chain fatty acids; JA, jasmonic acid; KCS, β-keto acyl-coA synthase condensing enzyme; KCR, β-keto acyl-coA reductase; ECR, enoyl-coA reductase; HCD, β-hydroxyacyl-coA dehydratase; FAR, fatty acyl-coA reductase; WS, wax synthase; LCBs, long chain bases; FAE, fatty acid elongase; MAH, mid-chain alkane hydroxylase; SPT, serine palmitoyltransferase; SBH, sphingoid base hydroxylase; PCD, programmed cell death; IPCS, inositolphosphorylceramide synthase; HR, the hypersensitive response; ROIs, reactive oxygen intermediates; SPT, serine palmitoyltransferase; CERK, ceramide kinase; PM, plasma membrane; DIM, detergent insoluble membrane; LR, lipid raft; GFP, green fluorescent protein; cer, eceriferum (not wax-carrying)

Key words: very long chain fatty acids (VLCFAs), plant-pathogen interactions, lipid signaling, sphingolipids, epicuticular waxes, lipid rafts, cuticle, plant defense

Recent findings indicate that lipid signaling is essential for plant resistance to pathogens. Besides oxylipins and unsaturated fatty acids known to play important signaling functions during plant-pathogen interactions, the very long chain fatty acid (VLCFA) biosynthesis pathway has been recently associated to plant defense through different aspects. VLCFAs are indeed required for the biosynthesis of the plant cuticle and the generation of sphingolipids. Elucidation of the roles of these lipids in biotic stress responses is the result of the use of genetic approaches together with the identification of the genes/proteins involved in their biosynthesis. This review focuses on recent observations which revealed the complex function of the cuticle and cuticle-derived signals, and the key role of sphingolipids as bioactive molecules involved in signal transduction and cell death regulation during plant-pathogen interactions.

Introduction

In response to pathogen attack, plants have evolved various mechanisms, both constitutive and inducible, in order to defend themselves. During the past 10 years, a number of studies have revealed the role of lipids and lipid metabolites during plant-pathogen interactions: (i) through the lipoxygenase pathway with the production of oxylipins and for example jasmonic acid (JA), which are important signaling molecules for defense regulation,[1,2] (ii) through the unsaturated fatty acid pathway by the remodeling of membrane lipid composition and defense signaling,[3] and finally (iii) through the very long chain fatty acid (VLCFA) pathway. The VLCFAs are fatty acids containing 20 to 36 carbons synthesized in the endoplasmic reticulum, which are crucial for a wide range of biological processes in plants. Their role in water-loss control or organ shape has been well studied. These lipids are indeed required for the biosynthesis of the plant cuticle,[4,5] and the generation of sphingolipids,[6,7] which can be bioactive molecules on their own.[8,9] This review focuses on recent studies that highlight the involvement of VLCFAs and VLCFA derivatives in the response of plants to pathogen attack. It will first describe the general pathway and the regulation of VLCFA and VLCFA derivative biosynthesis; then the role of these lipids in cell signaling and pathogen resistance through different aspects will be discussed.

*Correspondence to: Dominique Roby; Laboratoire des Interactions Plantes-Microorganismes (LIPM); UMR CNRS-INRA 2594/441; BP 52627; Castanet-Tolosan Cedex 31320 France; Tel.: 33.561.28.55.11; Fax: 33.561.28.55.61; Email: Dominique.roby@toulouse.inra.fr

Submitted: 12/09/08; Accepted: 12/10/08

Previously published online as a *Plant Signaling & Behavior* E-publication: http://www.landesbioscience.com/journals/psb/article/7580

VLCFA Biosynthesis, Regulation and Use for Wax and Sphingolipid Production

The elongation of the C16 and C18 fatty acids into VLCFAs (C20-C36 chains) takes place in the endoplasmic reticulum. Elongase complexes consisting in four enzymes mediate this step: a β-keto acyl-coA synthase condensing enzyme (KCS), a β-keto acyl-coA reductase (KCR), an enoyl-coA reductase (ECR) and a β-hydroxyacyl-coA dehydratase (HCD). Three different pathways then lead to the transformation of VLCFAs: in the epidermis, (i) through the "acyl-reduction pathway", primary alcohols and wax-esters are formed by fatty acyl-coA reductases (FAR) and wax synthases (WS), respectively, (ii) in the "decarbonylation pathway", VLCFAs are reduced to alkanes, which in stems are oxidised by mid-chain alkane hydroxylase (MAH1) to form secondary alcohols and ketones (Fig. 1, reviewed in ref. 5). Finally, (iii) in all cells, VLCFAs and long chain bases (LCBs) (generated from C16 and C18 fatty acids) are the precursors for the synthesis of sphingolipids[9] (Fig. 1).

The identification of the genes encoding VLCFA biosynthetic enzymes has been achieved mainly through mutational approaches. FAE1 (fatty acid elongase 1) was the first identified enzyme by the

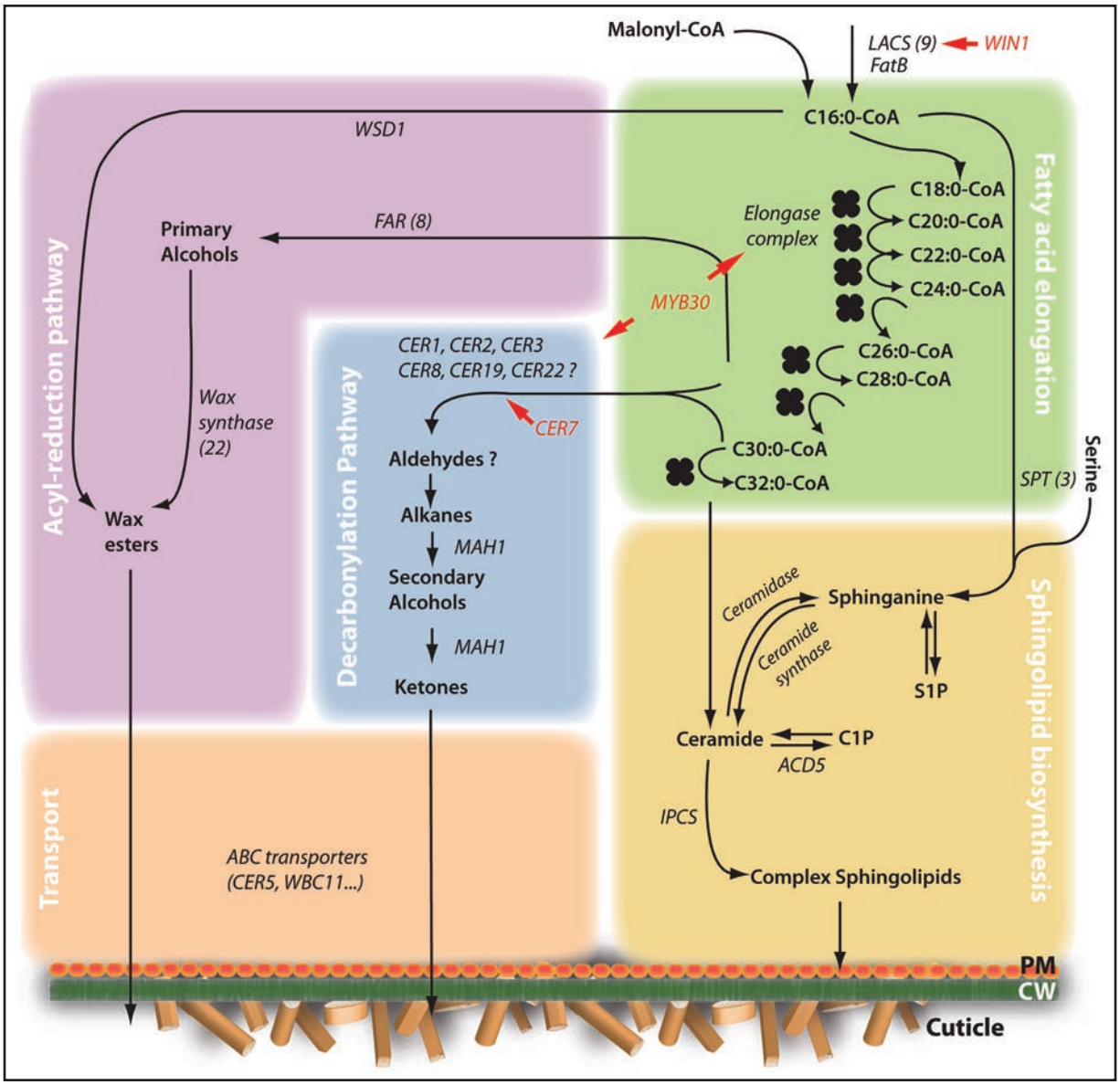

Figure 1. Simplified pathways for VLCFA and VLCFA derivative biosynthesis and transport in Arabidopsis. Regulators of these pathways and their putative action are indicated in red. PM: plasma membrane; CW: cell wall.

isolation of the *fae1*mutant altered in VLCFA accumulation in seeds.[10] Several *KCS* genes have also been identified through wax or cuticle mutants,[11-14] then by homology searches. They belong to a large gene family in Arabidopsis with 21 members,[15,16] hypothesized to drive the specificity (chain length, tissue…) of the elongation reaction. Concerning the other enzymes of the elongase complex, all of them have been recently found in plants, and in contrast to KCS, they are encoded by single genes: *AtYBR159* codes for the first reductase,[17] PAS2 revealed to be a 3-hydroxacyl-CoA dehydratase[18] and *CER10*, the homologue of the yeast gene *TSC13*, encodes the trans-2,3-enoyl-CoA reductase.[19]

Concerning wax biosynthesis, most of the genes have been screened by forward genetics in wax-deficient (*cer*) mutants. In the "acyl-reduction pathway", CER4 is the major fatty acyl-CoA reductase responsible for primary alcohol formation[20] while WSD1 produces wax esters in Arabidopsis stems.[21] In the "decarbonylation pathway", CER1 and CER3 are involved in alkane synthesis[22,23] while secondary alcohols and ketones are produced by the mid-chain alkane hydroxylase MAH1.[24] Finally, ABC transporters such as CER5 and WBC11 were shown to be involved in the transport of wax constituents through the plasma membrane.[25,26]

This is only recently that the genes controlling the biosynthesis of plant sphingolipids have been identified. In Arabidopsis, the gene *AtLCB1*, encoding the first subunit of the serine palmitoyltransferase (SPT), has been recently characterized.[27] The second subunit of SPT was also identified; however, 2 genes (*AtLCB2a* and *AtLCB2b)* encode functional isoforms of the LCB2 subunit.[28] Recently, two sphingoid base hydroxylase genes (*SBH1* and *SBH2*) have also been identified in Arabidopsis,[29] together with an inositolphosphorylceramide synthase[30] and a ceramidase from rice (OsCDase).[31] This enzyme, localized in the ER, catalyses the formation of phytoceramide.

Regulation of these biosynthesis pathways remains largely unknown. We have recently shown that AtMYB30, a MYB transcription factor, behaves as a transcriptional activator of several genes

encoding the four enzymes forming the fatty acid elongase complex, responsible for VLCFA biosynthesis.[32] Interestingly, this regulator acts as a positive regulator of a form of programmed cell death (PCD) in plants, the Hypersensitive Response (HR).[33] Concerning wax synthesis, the only transcription factors known to affect this pathway belong to the WAX INDUCER (WIN)/SHINE family in Arabidopsis.[34] The WIN1/SHN1 transcription factor activates genes encoding cutin biosynthetic enzymes for instance LACS2, and wax biosynthetic genes. However, the control of wax formation by WIN1/SHN1 may be indirect and may require additional transcription factors acting downstream of WIN1/SHN1. Finally, a new regulatory pathway controlling cuticular wax accumulation was recently discovered in Arabidopsis.[35] The key component of this pathway is the CER7 ribonuclease, a core subunit of the exosome involved in RNA processing and degradation. The putative target of this ribonuclease is an mRNA encoding a repressor of transcription of the key wax biosynthetic gene *CER3*.

Plant Sphingolipids as Key Signals during Plant-Pathogen Interactions?

Recent studies indicate that sphingolipids, as in animals, may play in plants a major signaling role in diverse fundamental processes. In terms of biotic stresses, the fungal *Alternaria alternata f. sp. lycopersici* (AAL) toxin has been shown to trigger cell death by disruption of sphingolipid metabolism.[36] Tomato plants sensitive to AAL-toxin accumulate more sphingolipid precursors due to a mutation in the *Asc* gene, encoding a component of the key enzyme ceramide synthase. Treatment with another mycotoxin, fumonisin, which is a specific inhibitor of ceramide synthase, leads to a dramatic accumulation of LCB and LCB-P in plant tissues. Lack of AtDPL1 lyase activity in the mutant lines enhances sphingolipid precursors accumulation and exacerbates fumonisin toxicity.[37] An Arabidopsis mutant, which is fumonisin B1 resistant (*fbr 11-1*), fails to generate reactive oxygen intermediates (ROIs), and cannot initiate PCD when the mutant is challenged by fumonisin B1.[38] *FBR11* encodes a long-chain base 1 (LCB1) subunit of serine palmitoyltransferase (SPT).[39] Consequently, free sphingoid bases are involved in the control of PCD in Arabidopsis, presumably through the regulation of the ROI level upon receiving different developmental or environmental cues.[38]

Another evidence in favor of the role of sphingolipids in the control of cell death in the context of plant-pathogen interactions has been the characterization of the mutant *accelerated cell death 5 (acd5).* The corresponding gene encodes a ceramide kinase (CERK)[40] and the mutant exhibits spontaneous cell death, shows enhanced disease symptoms during a pathogen attack and accumulates CERK substrates. This suggests that the balance between the ceramides and their phosphorylated derivatives are involved in the modulation of PCD and in the control of disease susceptibility. As previously mentioned, AtMYB30 has been shown to be a positive regulator of the hypersensitive response, through activation of VLCFA biosynthetic genes and increased sphingolipid production,[32,33] suggesting that sphingolipids would act as pro-cell death signals. The recent discovery of the gene *ERH1*, a gene acting as a negative regulator of the HR dependent on the resistance gene *RPW8* in response to powdery mildew infection, is also clearly in favor of a role of sphingolipids in the control of cell death and resistance in plants. Indeed, this gene encodes an inositolphosphorylceramide synthase (IPCS), and is rapidly induced during a pathogen attack, suggesting that it serves to increase GIPC (glucosyl inositolphosphorylceramide) biosynthesis for a defense related function.[30]

Together, these results show that multiple steps of the sphingolipid biosynthetic pathway are activated by infection, and that programmed cell death and disease resistance are in many cases tightly associated with this regulatory process. LCBs, ceramides and their derivatives might be the critical messengers to control cell death, or other defense mechanisms. Consequently it will be determinant to identify the targets of these bioactive sphingolipids to understand these regulatory mechanisms.

Are Lipid Rafts All-Armed Battleships?

In addition to their possible direct role as signaling components, sphingolipids also count among the constitutive elements of the plasma membrane (PM). They are also hypothesized to play a role in plant defense signaling as such. Sphingolipids were indeed shown to be quantitatively predominant, together with sterols, in the detergent insoluble membrane (DIM) fraction of the PM from tobacco, Arabidopsis and Medicago.[41-43] The lipid raft (LR) model[44] assumes that because of this distinctive biochemical composition, DIMs are organized in vivo in microdomains, and that the dynamic exclusion/incorporation processes within these domains can regulate signaling events. Consistently, DIMs were found to have specific protein content.[45] Some proteins are predominantly DIM-associated like remorins[46] or known components of plant defense responses such as the NDR1 GPI-anchored protein, suggesting the involvement of LRs in defense signaling cascades.[47]

Although direct involvement of LRs in plant defense responses has not been clearly evidenced to date, the cell biology of various plant-pathogen interactions is consistent with the LR theory. Polarization of the cytoskeleton, aggregation of peroxisomes, endoplasmic reticulum and Golgi bodies at the interface with the pathogen are among the first observable responses of a plant cell to a pathogen attack (reviewed in ref. 48). In addition, polarization and focal accumulation processes are not only found inside the cell but also inside the PM itself. Barley and Arabidopsis cells challenged by the powdery mildew pathogen *Blumeria graminis*, were shown to undergo focal accumulation, beneath the site of appresorium formation, of a subset of GFP-fused PM proteins (the resistance locus O, the protein MLO, the ROR2 syntaxin and its Arabidopsis ortholog PEN1), whereas other PM proteins (aquaporin isoforms, SYP132 syntaxin) were not re-localized upon infection.[49] Similarly, the GFP-fused ATP-binding cassette transporter PEN3/PDR8 localizes in the PM of Arabidopsis un-inoculated leaves and showed strongly focused accumulation at sites of Blumeria attempted penetration.[50]

However, if experimental evidences for filamentous pathogen-induced focal protein clustering at the PM are emerging, the underlying mechanisms remain enigmatic. On the one hand, the filipin antibiotic, which binds sterols, shows enhanced labelling at Blumeria entry sites, suggesting aggregation of plant LRs or the release of sterol-rich fungal material.[51] On the other hand, the hexose-proton symporter HUP1 was shown to segregate in the DIM fraction and to show a sterol-dependent spotty distribution in the PM when expressed in yeast.[52] These results suggest the existence of detergent-insoluble PM domains in planta. Nevertheless, lateral organization of

membrane lipids could not be clearly associated to pathogen-induced protein focal accumulation events to date. Therefore, the precise role of PM domain clustering in plant defense responses remains poorly documented. In addition, it is still not known whether membrane focal accumulation is restricted to response toward filamentous pathogens or to certain types of plant-pathogen interactions.

The Plant Cuticle, More than a Protective Shell against Pathogens

Well-organized cuticle layers, made of cutin and wax polymers, covers aerial plant surfaces. In many plant species, cutin originates from polymerization of C16 and C18 ω-hydroxylated fatty acids. By contrast, wax synthesis requires elongation of C16 and C18 fatty acids into VLCFAs. These compounds form the outermost layer in epidermal cells, and therefore the first barrier encountered by pathogens in the natural environment. Nevertheless the impact of plant cuticle on the outcome of the interaction with a pathogen is beyond a simple barrier effect.

First, the cuticle can be considered as a reservoir of signals telling phytopathogenic fungi that they found a proper host to infect. This hypothesis originates from the observation that fungi often secrete cutinases when reaching a plant, and that cutin monomers induce appresorium formation in *Magnaporthe grisea* and *Erysiphe graminis*.[53,54] Consistently, removal of cuticular waxes reduces conidial germination of *Blumeria graminis* on barley,[55] and the altered cuticle of the *sma4* (allelic to *bre1* and *lacs2*) Arabidopsis mutant inhibits *Botrytis cinerea* spore germination.[56] Therefore, a thinner, more permeable cuticle does not facilitate the entry of these pathogenic fungi but rather arrests their invasion.

In addition to being detrimental to the growth of the pathogen, cuticle alteration also favors the onset of defense by the plant, as plants can perceive modifications of the cuticle. Exogenous expression of a fungal cutinase gene in Arabidopsis provides immunity to *B. cinerea* due to the release of fungitoxic compounds and activation of other resistance-associated genes. Similarly, release of antifungal compounds and enhanced *B. cinerea* resistance have been observed in the *bgd* mutant that exhibits cuticular defects.[57] Increased release of antifungal compounds also plays a role in the enhanced resistance to Botrytis and Sclerotinia observed in *sma4* mutant.[58] Nevertheless, a positive effect of the cuticle on defense processes is also documented. The plant cuticle not only protects from water loss, but also reduces plant-pathogen recognition and efficient release of antifungal compounds in certain cases during the infection process. Double knockouts *gpat4/gpat8*, with strongly reduced cutin content, are less resistant to *Alternaria brassicicola*.[59] Whereas more resistant to *B. cinerea*, the *sma4* mutant shows a normal susceptible phenotype toward *Erysiphe cichoracearum*.[56] These two examples illustrate the current consensus that cuticle thickness has a negative effect on biotrophic fungal growth. The *att1* mutant has a cutin content reduced by 30%, and shows enhanced susceptibility to virulent *Pseudomonas syringae*.[60] This mutant causes enhanced expression of bacterial type III genes, suggesting that cutin-related compounds repress bacterial type III genes expression in the plant apoplast. On the other hand, *att1* displays enhanced resistance to *B. cinerea*.[56]

Taken together, these data essentially based on analysis of cuticle-defective mutants, indicate that cuticle-derived signals act negatively on necrotrophic fungal infection and positively on biotrophic fungi and virulent bacterial pathogens. However, further investigations involving gain-of-function experiments and various plant-pathogen interactions will be required before reaching a general rule on the contribution of cuticle related genes and cuticle composition to plant defense mechanisms.

Conclusions and Perspectives

Within the past few years, there has been a "burst" of articles in the field of plant pathology which have revealed the various and major roles of lipids, and more recently, the function of very long chain fatty acid-based molecules during plant-pathogen interactions (Fig. 2). Identification of the roles of these lipids in biotic stress responses is the result of the use of genetic approaches together with the rapid progress made in the identification of the genes/proteins involved in lipid biosynthesis. These discoveries highlight the complex function of the cuticle and cuticle-derived signals, and the key role of sphingolipids as bioactive molecules involved in signal transduction and cell death regulation. However, the results are still fragmentary and need further investigations.

Sphingolipids are known to function in all eukaryotic cells as membrane structural and signaling components. Intensive research in the animal field has shown that these compounds play essential regulatory roles in the control of cell death and cell survival.[9,61,62] A number of reports described here suggest that plant sphingolipids might be involved in similar regulatory mechanisms. However, this notion that there may be common lipid signaling mechanisms for the control of cell death in plants and in animals often relies on indirect or correlative evidences.[63-65] Besides, these compounds being active in low amounts and existing under a large number of different molecular forms, studies on their biochemistry are still limited. Extraction, separation and identification of such compounds remain a challenge, and an accurate picture of the different species and biosynthetic pathways of plant sphingolipids is not yet available. The systematic identification of sphingolipid biosynthesis genes will also face the difficult task of assessing their enzymatic/molecular function and of the identification of their targets. Together with a genetic approach to addressing the question of their importance in defense/cell death signaling, these are keys to understand the role of sphingolipids in plant cell signaling.

After intensive studies in animal systems, lipid rafts start appearing as ubiquitous entry sites for pathogens, in plants as well. Again, the gap between animals and plants in term of PM raft functions should not be filled in prematurely. But in this case, biochemistry is ahead of genetics, and assessing the existence and function of PM microdomains in vivo is a major expectation. A key step toward this achievement could be the identification of plant counterparts of caveolins, or flotillins, that is to say protein markers of lipid rafts that allow detection of rafts in living cells. Pathogen attack would then be a convenient stimulus to study raft dynamics and role in signaling.

Finally, epicuticular waxes are specific to plants, known to play important general functions in the interactions of plants with their environment.[66] This key adaptation in the evolution of plants[67] has been shown to influence the issue of plant-pathogen interactions in unexpected ways. This is clearly an exciting area of research, and the important question of the signaling function of some components of the cuticle will need future work, using different plants, pathogens and adequate biochemical methods to identify the active molecules

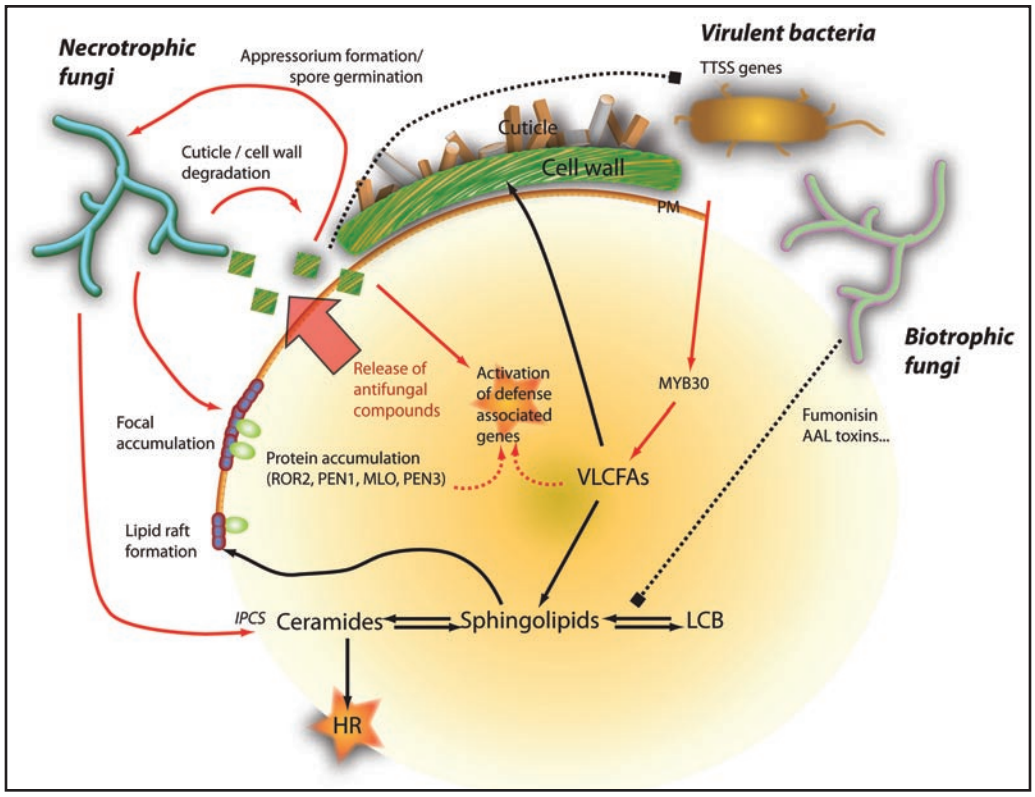

Figure 2. Schematic diagram summarizing the contribution of VLCFAs and VLCFA-derived molecules to the molecular dialogue occurring during plant-pathogen interactions. Exchanges between the plant and different pathogens involve (i) VLCFAs and sphingolipid synthesis, (ii) cuticular compounds, and (iii) PM microdomain organization. As a consequence, pathogen growth is differentially affected by the onset of the HR, according to their invasion strategy (e.g., positively for necrotrophic fungi, negatively for biotrophic fungi). Plain red arrows indicate a positive effect; dotted lines with boxed arrowhead, a negative effect, dotted red arrows shows putative positive effect. PM, Plasma Membrane; TTSS, Type Three Secretion System; LCB, Long chain Base; HR, Hypersensitive Response.

and their exact roles. Another intriguing question is related to the putative signaling roles of LTPs (Lipid Transfer Proteins). In favour of this hypothesis is the identification of DIR1, a putative LTP involved in the long distance signaling associated with systemic resistance.[68] DIR1 has been recently structurally characterized, sharing some structural and lipid binding properties with LTP2, but displaying some specific features.[69] A functional analysis of this protein family in Arabidopsis would bring some light on lipid signaling and transport. Finally, in depth analysis of the functions of VLCFAs and VLCFA derivatives during plant-pathogen interactions will undoubtedly provide access to fundamental functions of these compounds during plant development.

Acknowledgements

We are very grateful to Dr. Frederic Domergue for critical reading and comments on the manuscript.

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